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II. EVALUATION OF PHOTOTOXICITY OF SALICYLANILIDES AND SIMILAR COMPOUNDS BY PHOTOHEMOLYSIS*

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ABSTRACT

A system is described in which red blood cell photohemolysis is induced by salicylanilides and similar compounds. The range of chemical and physical conditions at which each compound reacts defines its ability as a photosensitizer. Results are correlated with the known clinical photoactivity of these compounds.

Pre-irradiation by artificial and natural sunlight produced photoproducts which were evaluated by thin layer chromatography, ultraviolet spectroscopy, osmotic fragility, pH changes and toxicity to red blood cells.

Salicylanilides and their congeners have become the most common cause of topical photosensitization from household products and soaps. For diagnosing patients photoallergic to these agents the photo patch test has become an established procedure. However, there are no established *in vitro* tests that assess the potential phototoxic dangers of salicylanilides. All current methods for predicting photoactivity of salicylanilides are *in vivo*, employing the cutaneous system (1–3). This report is of an *in vitro* method for predicting the phototoxic potential of salicylanilides and similar compounds.

A previously described *in vivo* test for photoallergy used tape stripped human skin onto which 10% TCSA† was applied for several days before eliciting reactions (1). Another test photosensitized epilated guinea pigs readily with TCSA and bithionol but only "under conditions of extreme stress" with TBS and TCC (2–3). A test for phototoxicity using intraperitoneal injections of salicylanilides into hairless mice failed to photosensitize (4), as did our attempt to evaluate salicylanilide photosensitivity *in vitro* with *Candida albicans*, (unpublished data, G. Kahn). Photoeffects of salicylanilides on cell cultures have not been reported.

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† Abbreviations:
TCSA 3,3′4′5 tetrachlorosalicylanilide
TBS 3,4′5 tribromosalicylanilide
DBS 3,5 or 4′5 dibromosalicylanilide
MBS 4′ monobromosalicylanilide
TCC 3,4,4′ trichlorocarbanilide

To establish an *in vitro* test which defined the phototoxic tendencies of these substances quickly and reproducibly, we modified a photohemolytic test system which Oleniacz, *et al.* used to induce photohemolysis with tetrahalogenated salicylanilides (5). Oleniacz, *et al.* did not induce photohemolysis in solutions of MBS, DBS or TBS.

In our method we use red blood cells (RBCs), buffered solutions, and organic solvents with the tested compounds. Solutions are irradiated 30–90 minutes with a Westinghouse FS-40 sunlamp. Results are reproducible.

We evaluate the phototoxic capabilities of halogenated salicylanilides and of chemically similar known photosensitizing agents. It is emphasized that this test is not a measurement of photoallergy. Our initial report shows that under appropriate conditions most photosensitizing drugs cause photohemolysis in our system (6).

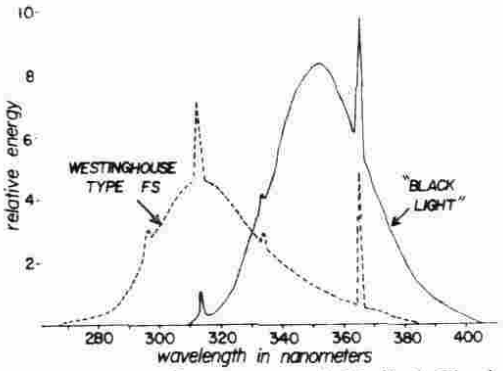
METHODS AND MATERIALS

Ultraviolet light sources used: (see Fig. 1)	Energy output‡ onto solutions	Spectra in nanometers
General Electric F1ST8B1b black lamp	1×10^4 ergs/cm²/sec	320–400 (Fig. 1)
Westinghouse FS-40 sunlamp	1×10^4 ergs/cm²/sec	280–370 (Fig. 1)
General Electric G15T8 bacteriocidal lamp	1×10^4 ergs/cm²/sec	254
Sunlight	$\sim 2 \times 10^4$ ergs/cm²/sec	295 to infrared

‡ Output was measured by a YSI Kettering model 65 radiometer.

Photohemolysis. Salicylanilides were dissolved in methanol, then diluted to final concentration in veronal (barbital) buffer at pH 7.4 TCC was dissolved in ethanol and hexachlorphene and MBS were dissolved in acetone. Organic solvents promoted solubility of the tested chemicals; an equal amount of solvent was added to "control" solutions.

Packed human RBCs, washed three times in physiological saline, were added to the buffer and chemical solution (0.2% v/v) and mixed gently. The solution was poured into Carrel tissue culture flasks and exposed to the sunlamp at 37°C for 30 or 90 minutes. Identical flasks were kept in the dark at 37°C as controls.



From the Department of Medical Physics, Temple University Medical School, Philadelphia.

FIG. 1.

After ultraviolet (UV) exposure or incubation in the dark, the solutions were centrifuged at 2000 RPM. The optical density (OD) of the supernatant fluid, read at 540 nm on a Beckman DU-2 spectrophotometer, measured hemoglobin released from hemolyzed RBCs. A total hemolysis control was prepared by adding 0.02 ml of the washed RBCs to 10 ml of a 0.04% NH_4OH solutions. Results were expressed as % hemolysis by using the following formula:

$$\frac{\text{OD exposed solution} - \text{OD dark control}}{\text{OD total hemolysis control}} \times 100$$

Pre-irradiation. Based on the results of other investigations (17–19) compounds were pre-irradiated by the above mentioned light sources 18 to 72 hours. Irradiation occurred in veronal buffer, in 1% aqueous ethanol and in petrolatum. Solutions exposed to the GE germicidal lamp were placed into open beakers and were restored to original volume after irradiation.

Changes in the absorption curves of the salicylanilide derivatives, produced by UV irradiation were recorded by a Cary spectrophotometer, model 15. Changes in the osmotic fragility of the RBCs before and after irradiation, with and without drugs, were recorded by a Fragiligraph (Kalmedic Instruments, N.Y., N.Y.).

To determine the isolated effect of UVL on the drug or on the RBC, the following three hour dark incubation experiments were done at 37°C:

a. pre-irradiated RBCs with non-irradiated drug
b. pre-irradiated drug with non-irradiated RBCs
c. pre-irradiated drug with pre-irradiated RBCs

Thin layer chromatography: Thin layer chro-

TABLE I

Range of activity of compounds

	Concentration in dispersing		Solvents	Spectrum
	mg %	medium		
TCSA	0.1–LS*	All†	All‡	Very wide
TBS	0.1–LS	All	All	Very wide
DBS	1–LS	All	All	Wide
Bithionol	.1–.5	All	All	Medium
4′ MBS	5–LS	Barbital citric acid (pH 8)	Acetone	Narrow
Hexachlorophene	0.1–0.5	All	Acetone	Narrow
TCC	0.5–1	Barbital buffer	Ethanol	Narrow
MB salicylic acid and DB salicylic acid	5–20	Only tested in methanol and barbital buffer	—	—

* L.S.—limits of solubility

† All (media)—physiological saline, phosphate buffers (0.1 + .5 M), veronal (barbital) buffer, citric acid buffer

‡ All (solvents)—methanol, ethanol, acetone

This table uses "range" to define the chemical spectrum of conditions in which a substance will photohemolyze RBCs. The greater the "range", the greater the phototoxicity. Note that concentration is not the only determinant of photoactivity.

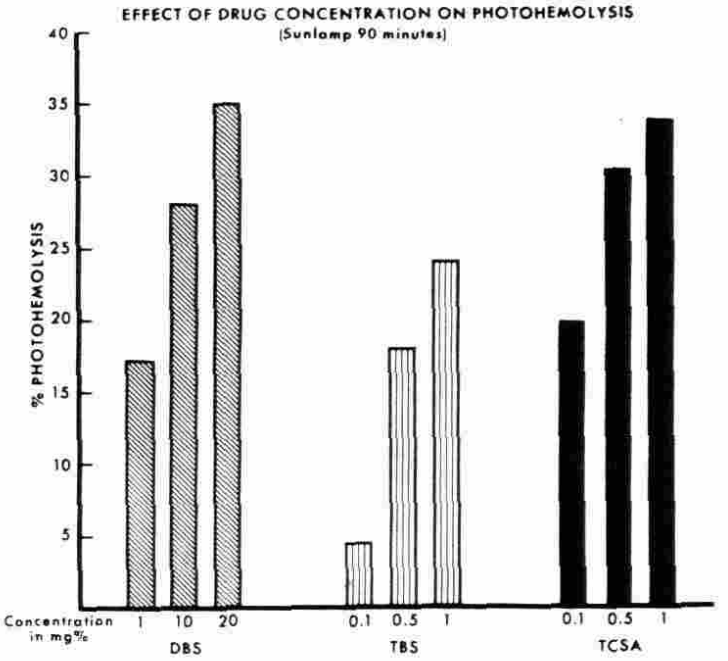


FIG. 2. TCSA is more active than TBS. At 1 mg. % both are more active than DBS.

TABLE II

Salicylanilide photosensitivity

Clinical vs. *In vitro* i.e. Photoallergy vs. Phototoxicity

Clinical	*In vitro*
TCSA	TCSA
DBS	TBS
TBS	DBS
Bithionol	Bithionol
4′ MBS	4′ MBS
Hexachlorophene	Hexachlorophene
TCC	TCC

The *in vitro* activity is based on data taken from Table I. The *in vivo* activity is based on the combined clinical reports of several investigators.[9-14] Drugs are listed in the order of reactivity. TCSA and 4′ MBS are not present in commercial products, minimizing the clinical risk of exposure to them.

matography (TLC) was performed by the method described by Wolfrom *et al.* (7) except that we used a new type of pre-coated aluminum, microcrystalline cellulose plate (S18952-30 Merck) (8). The plates, which resolved closely related isomers, could be handled without special precaution, making them suitable for routine use.

Salicylanilide solutions (1.0 mg %) were centrifuged 10 minutes at 1600 g. The precipitate was dissolved in 5 ml methanol and applied in 20 ul spots which were eluted with methanol:H_2O: acetic acid, 50:50:1. Plates were examined under UV light (240–400 nm). Similar studies were performed on salicylanilide residues of petrolatum into which 1 gram % salicylanilide powder had been triturated. The petrolatum-salicylanilide was separated by three extractions in a 50 cc hexane—

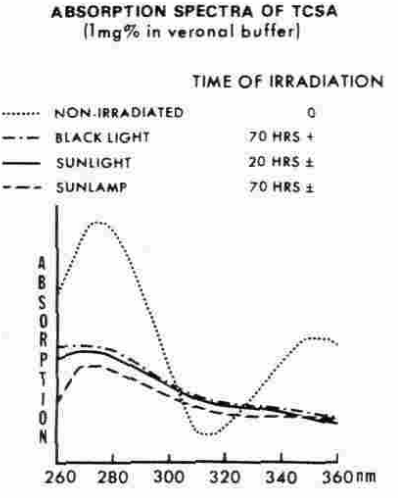


FIG. 3. UV irradiated salicylanilides produced new absorption spectra with all light sources used.

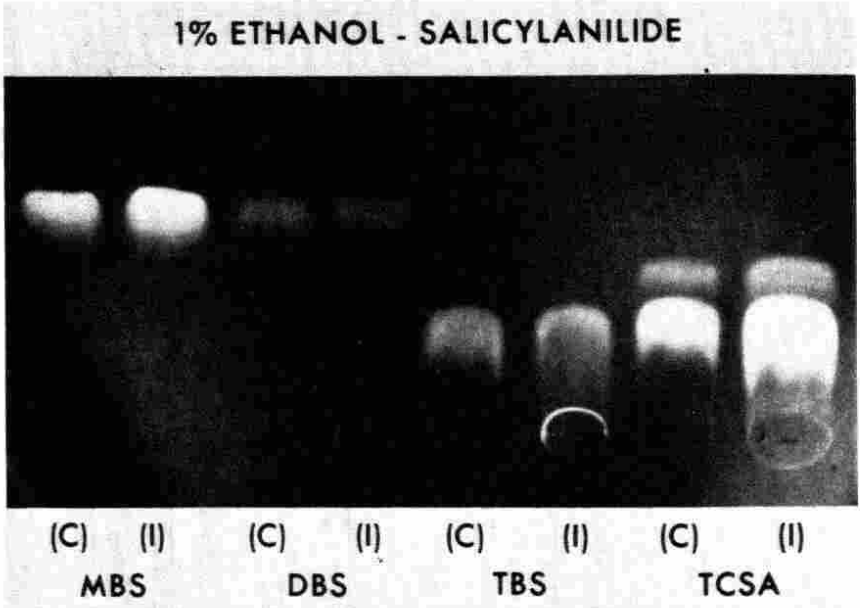


FIG. 4. Fluorescent rings (photoproducts) were produced by irradiation (I) of controls (C). This effect is noted least with MBS (control is blackened from camera leak). TCSA (C) is impure.

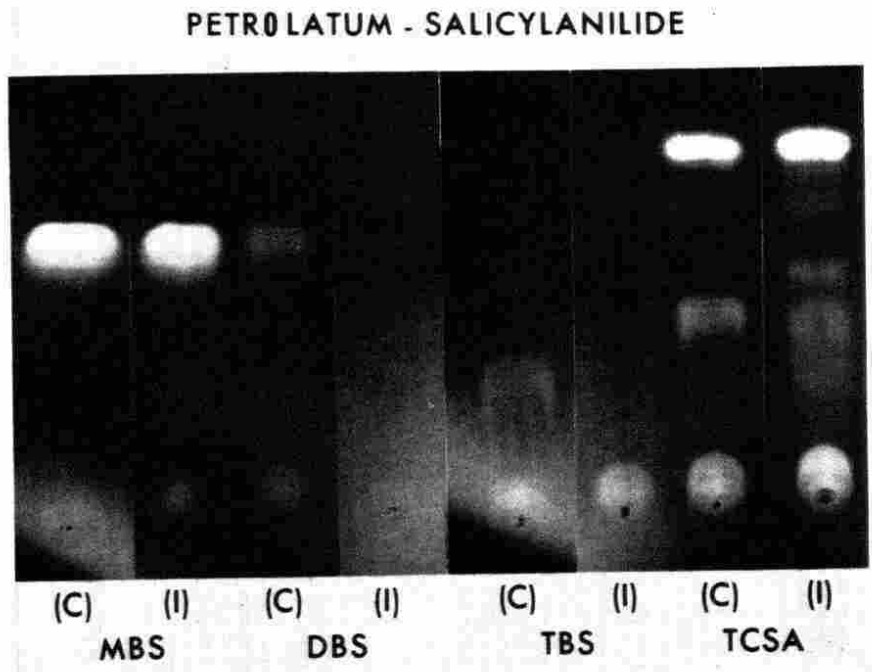


FIG. 5. Variegated changes produced by irradiation (I) in petrolatum: MBS changed least; faint fluorescent spot produced, was not demonstrated on photograph. DBS and TBS lost fluorescence. TCSA (impure) formed several fluorescent products.

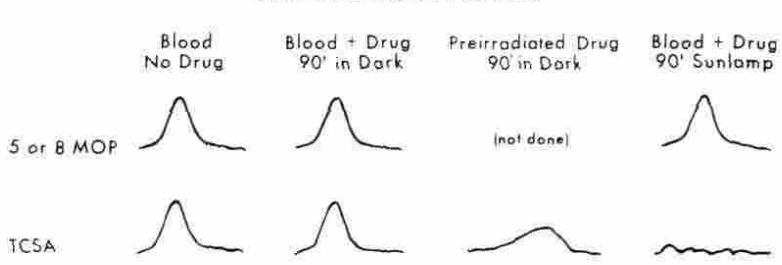


FIG. 6. Osmotic fragility curves comparing psoralens (non-reactive) to salicylanilides (reactive). Salicylanilides affect RBCs in the presence of light, but not in the dark. Photoproducts (pre-irradiated salicylanilides) affected cells even in the dark.

5 cc methanol mixture. The methanol was removed from the salicylanilide residue by vacuum suction.

RESULTS

Our results indicate that TCSA and TBS photohemolyze RBCs in all chemical conditions used in our system and to a wider range of chemical conditions than any other agents tested (Table I). TCSA is more active than TBS (Fig. 2) (6).

Though some compounds are very reactive at low concentrations, their range of reactivity is very narrow, i.e. MBS, hexachlorophene and TCC hemolyze only in the presence of selected buffers and organic solvents. TCC must be dissolved in ethanol, while MBS and hexachlorophene work only if dissolved in acetone.

Of intermediate hemolytic activity were DBS, bithionol, and the salicylanilide derivatives, 3 and 3,5 bromosalicylic acid.

We were somewhat able to relate the activity of salicylanilides and congeners to that which has been reported clinically by several investigators (Table II) (9–14).

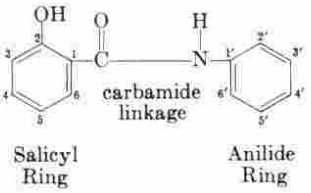
Salicylanilides, irradiated with all light sources, caused changes in the absorption spectra (Fig. 3) and produced new photoproducts, which were confirmed by thin layer chromatography. MBS, DBS, TBS and TCSA changed with all light sources used. MBS caused the least spectrophotometric or chromatographic changes. Photographs of chromatograms did not demonstrate the small MBS changes (Figs. 4,5). When salicylanilides were irradiated in petrolatum, changes appeared greater than those seen in alcoholic or buffered solutions. In alcoholic solutions new fluorescent rings were uniformly noted at the spots of origin, while variegated changes were produced in buffer solution and in petrolatum. Our TCSA was proved impure by TLC. The pH of buffers changed from 7.4 before irradiation to 7.2 after irradiation.

Osmotic fragility studies revealed that salicylanilides acted upon RBCs in the presence of light, but not in the dark. Pre-irradiated salicylanilides, however, changed the osmotic fragility curve even in the dark (Fig. 6). Irradiation of salicylanilides in the presence of RBCs affected the cells markedly, as illustrated by the flattened out derivative curve of Figure 6, which compares the RBC fragility of psoralens (non-reactive) to that of salicylanilides (reactive).

When pre-irradiated salicylanilides are combined with non-irradiated RBCs or when pre-irradiated RBCs are combined with non-irradiated salicylanilides minimal hemolysis occurs (5–10%). We found that pre-irradiation of both RBC and drug separately for 90 minutes by the sunlamp, followed by 3 hours of dark incubation with drug and cells combined, caused more hemolysis than conventional photohemolytic methods (6). This unexpected finding occurred because irradiation of salicylanilides produced photoproducts with greater toxicity to RBCs than the parent compound. MBS showed this toxic effect less than other salicylanilides. In contradistinction, pre-irradiation of bithionol and hexachlorophene produced photoproducts considerably less toxic to RBCs than the parent compound.

DISCUSSION

Antibacterial agents characterized by a phenyl-carbamide linkage have been used as broad



spectrum antiseptic agents for many years (15–16). The mildness and stability of some brominated salicylanilides used in aerosols, surface disinfectants and soaps vaulted them into a prominent place as germicidal agents in industrial and domestic markets in the 1960's. Though the salicylanilides are stable to heat, they are rather selectively sensitive to UV light, and structural changes occur when light is absorbed by these substances.

Irradiation of ethanolic-salicylanilide solutions and the resultant photoproducts have been characterized previously (17–19). Initially a halogen is removed from the three position of the salicyl ring and thereafter from the five position (18). It is stated that the 4′ position of the anilide ring is immune to changes from UV light (18). However, our spectroscopy, TLC and cell toxicity data show that 4′ MBS is changed by UVL.

Thin layer chromatography by Baker of ethanolic and aqueous solutions revealed fluorescent products from irradiated salicylanilides (19). We confirmed these observations using 1% methanolic-aqueous solutions. We also performed chromatography after irradiating salicylanilides in barbital buffer and in petrolatum. Greater changes were produced in petrolatum. The changes produced on TLC were correlated with changes in spectroscopic absorption of newly formed photoproducts.

The photoproducts of salicylanilides were more toxic to RBCs in the dark than their parent compounds. Curiously, bithionol or hexachlorophene photoproducts were non-toxic to cells.

It is not known if salicylanilides accruing on the skin are changed in the same way as when light strikes them in an in vitro solution or mixture. It has been suggested that the products formed in petrolatum can cause the skin of sensitized patients to react (20).

Photoreactions caused by salicylanilides are believed to result from the induction of free radicals which combine with cutaneous proteins to induce photosensitization. In addition, evidence of virtually all workers supports the premise that salicylanilides become photoreactive by triplet formation. Oleniacz *et al.* used triplet quenchers (KI, $NaCl_2$) to suppress salicylanilide photoreactivity (5).

Our buffer, ethanol and petrolatum systems may be similar to each other in that produced photoproducts were produced in all, identified by chromatography and spectroscopy, that were different from irradiated parent precursors. The absorption characteristics of photoproducts were not dependent upon the light source used for irradiation (Fig. 3, bactericidal lamp not depicted). In basic solutions most of the halogenated salicylanilides absorb wave lengths greater than 280–360 nm. These broad absorption spectra minimize the importance of the irradiation sources used in these experiments, because all of the used sources can produce photoproducts.

The lack of activity of the anilide ring in salicylanilide photoactivity was shown by Harber, who demonstrated positive photopatch tests on 4 of 19 patients to 3,5 dibromosalicylic acid and 5 bromosalicylic acid, agents which have no anilide rings (21). This is concordant with our results which showed that these salicylic halogens cause photohemolysis (Table I).

Under appropriate laboratory conditions, salicylanilides and derivatives which clinically cause photosensitivity also induce light-mediated permeability changes in red blood cell membranes. The extent of this phototoxic damage is measured indirectly by spectrophotometric absorption of the hemoglobin released. Measurements of damage to red blood cells reflect only the phototoxic actions of a drug. In a general way, we were able to quantitate the phototoxic potential of salicylanilides and other photosensitizing antiseptic agents.

The RBC has no organelles: therefore it provides a system in which compounds can be evaluated for their ability to interact with a single morphological component, the cell membrane. Our fragiligraphic studies with salicylanilides graphically illustrate the need for light to induce the interaction of the cell membrane with drug (Fig. 6). The chemical and physical nature of this interaction remains unknown.

Photohemolysis circumvents the use of intact, living skin for evaluating photosensitivity. Unlike other tests for salicylanilides, photohemolytic analysis takes hours rather than days. Before testing any substances, we determine the highest concentration of the compound that will not

hemolyze red blood cells in the dark. Using the appropriate buffer-solvent mixture, we then can screen the drug quickly at maximal concentration for phototoxic potential. This potential then can be compared with the results we have reported here with substituted salicylanilides and congeners to estimate the drug's relative toxicity and photoactivity.

The limitations of our system are that it cannot test substances severely toxic to RBCs, nor can it test contact sensitization or photoallergy. The rankings of phototoxicity come close to those of photoallergy in the known clinical situation i.e. TCSA is most photosensitizing. The most reactive agents hemolyze RBCs in a wide spectrum of test conditions and are the most effective and common photosensitizers in vivo; it is the narrow spectrum reactors that are the rare photosensitizers *in vivo*. Concentration and per cent hemolysis are not the main parameters that reflect clinical activity, but a broad spectrum of phototoxic activity under a variety of chemical conditions are the pre-requisites which prognosticate the clinical photoreactivity of a compound.

A quick, reproducible and easily performed *in vitro* test to determine phototoxic potential of compounds is needed to screen products for photosensitizing potential long before they reach the marketing arena. To achieve this for salicylanilides and similar compounds, we employ and recommend the described photohemolysis test.

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